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Studies focused on in vivo assessment of enhancing tumor response to radiation (XRT) and adriamycin by pretreatment with 6-aminonicotinamide (6AN) in the hormone sensitive MCF-7. Tumors were inoculated on the flank of nude mice and were studied when they attained a volume of ~ 150 mm³. The dose of 6AN for all experiments was 16 mg/kg which was given 10 hours prior to adriamycin or XRT (5 Gy). Mice were treated on days 0, day 11 and day 21. The pooled data from 3 experiments indicated a tumor regrowth delay (time to regrow to initial volume) of 21.8 +/- 3.2 days for mice treated with 5 Gy (n=26) vs. 53.7 +/- 4.0 days (n=26) for mice treated with 6AN→5 Gy, which was highly significant (p<<0.001). The partial response rate (>50% tumor shrinkage) for mice treated with XRT alone was 1/26 vs. 14/26 for mice receiving 6AN→5Gy. Tumor delay (time for tumor to increase by 50%) for mice treated with adriamycin (10mg/kg=maximum tolerated dose) was 33.2 +/- 2.9 days (n=12) vs. 47.2 +/- 5.2 days for mice receiving 6AN (16mg/kg) + adriamycin (n=14) (p<0.025). We have also begun studies on hormone resistant tumors as initially proposed. We have completed the NMR studies on cells using this tumor model. Perfused MDA-MB-435 cells are stable for periods of >50 hours while being perfused in the NMR system. In contrast, cells treated with 6AN (200mM) leads to a loss of phosphocreatine and nucleoside triphosphates, the detection of 6-phosphogluconate, an increase in inorganic phosphate (Pi) and splitting of the Pi peak into different components, indicating a change in pH.

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Introduction

The goal of the research conducted under this grant is to investigate the efficacy of 6-aminonicotinamide (6AN) to enhance the effect of radiation and chemotherapy agents that are used in treating breast cancer. This proposal was based on previous work that indicated that 6AN enhanced the effect of radiation in vitro (RIF-1 tumor cells)(1,2) and in vivo (3) (breast cancer tumor). The criticism of the latter research was that the studies had not been done in human tumors and therefore we undertook these studies to determine if it was possible to reproduce our previous results in human breast cancer cell/tumors. Our previous in vitro studies from year 1 indicated that 6AN did enhance the effect of radiation and adriamycin, but not paclitaxel (4).

Body

Studies in year 2 focused on in vivo assessment of enhancing tumor response to radiation and adriamycin by pretreatment with 6-aminonicotinamide (6AN) in the hormone sensitive MCF-7. Preliminary studies using the three drug regimen, PALA (Phospho-N-acetyl aspartate), MMPR (6-methylmercaptapurine riboside) and 6AN, were also done. In addition, we have begun studies on a hormone resistant tumor model using these drugs.

Tumor Growth Delay Studies (MCF-7)

Tumors were inoculated on the flank of nude mice and were studied when they attained a volume of $\sim 150 \text{ mm}^3$. In the early studies mice were injected when they were approximately 10 weeks old, although in later studies, younger mice (6-8 weeks old) were used. The tumors grow more rapidly in the younger mice and we have modified our technique to purchase mice when they are 6 weeks old and inject them with tumor cells within 1-2 weeks after delivery. Our experimental methods included running cohorts of controls (no treatment) on each experiment to allow for variations such as these. To evaluate tumor response, tumor growth (or regrowth after treatment) was measured. In experiments involving radiation (XRT), we chose to measure the time it took for the tumor to regrow to its initial tumor volume. We also measured complete response (complete disappearance of tumor) and partial response (decrease in tumor volume by 50%). For experiments with chemotherapy drugs only (no XRT), we measured the time required for the tumor volume to increase by 50%. Data are reported as mean \pm standard error of the mean (SEM). Tumor volume was measured as the product of $(d1 \times d2 \times d3)\pi/6$ where $d1$, $d2$, and $d3$ are three perpendicular diameters. Mice were treated on day 0, day 11 and day 21. The interval between 6AN and adriamycin or XRT was 10 hours. The dose of 6AN for all experiments was 16 mg/kg based on work accomplished in year 1 of the study. This was noted to be decreased compared to the CD8F1 mice which tolerated 20 mg/kg. Mice who died within the first 28 days of the study were censored from the data analysis.

We initially examined the effect of 6AN on radiation sensitivity. Three independent studies were done and the data are shown individually and combined. Tables 1-3 demonstrate that in all three experiments, pretreatment with 6AN enhanced the effect of 5 Gy. The activity of 6AN alone was evaluated by measuring the time for the tumor to increase by 50% since for both control and 6AN treated cohort, there wasn't any shrinkage or regrowth delay. 6AN is noted to have a modest anti-neoplastic effect as evaluated in Table 4 which presents the pooled data from all experiments. The major finding was that 6AN clearly enhanced response to XRT in all three experiments and in the combined data. Tumor regrowth delay were 39.7 ± 7.1 , $>55.0 \pm 6.4$ and $>68.7 \pm 2.2$ days for the three experiments. The ">" is due to the fact that several tumors have not yet regrown to their pretreatment tumor volume. In contrast, the response to 5 Gy was measured by tumor regrowth delays of 18.7 ± 6.4 , 23.7 ± 5.6 and 37.0 ± 4.3 days respectively. The pooled data indicated a tumor regrowth delay of 21.8 ± 3.2 days for 5 Gy ($n=26$) vs. $>53.7 \pm 4.0$ days ($n=26$) which was highly significant ($p < 0.001$). The partial response rate for mice treated with XRT alone was 1/26 vs. 14/26 for mice receiving 6AN—>5Gy.

We also examined the effect of effect of 6AN on response to adriamycin. In previous experiments with the CD8F1 tumor, the maximum tolerated dose (MTD) of adriamycin was 11 mg/kg on this schedule (3 doses over 3 weeks). However, nude mice are more fragile and the MTD was only 10

mg/kg (28 day mortality was 2/15 mice). The 28 day mortality of mice treated with 11mg/kg was 3/9. Tumor delay (time to grow by 50%) for mice treated with adriamycin (10mg/kg) (Table 5) was 33.2 +/- 2.9 days (n=12) vs. 47.2 +/- 5.2 days for mice receiving 6AN (16mg/kg) + adriamycin (n=14) ($p < 0.025$). The 28 day mortality for the latter group was 1/15 mice. A parallel cohort of mice treated with 6AN + adriamycin at 5mg/kg were also studied. Their 28 day mortality was 2/15 mice. Their time to regrowth was 36.8 +/- 5.4 day (n=13) demonstrating the importance of administering adequate doses of adriamycin.

NMR Cell Studies

We have begun studies on hormone resistant tumors as initially proposed. We had difficulty with growing the MDA-MB-231 (slow growth rate) so this model was not used. The MDA-MB-435 grew to 150 mm³ in 3-4 weeks and studies with this tumor model were therefore initiated. We have completed the NMR studies on cells using this tumor model. The methodology is identical to that published previously (1,2,4)

Figure 1 shows a control ³¹P NMR study demonstrating that the perfused MDA-MB-435 cells are stable for periods of >50 hours while being perfused in the NMR system. The spectral peaks are identified as B= phosphoethanolamine, C=phosphocholine, D=inorganic phosphate (Pi), E=glycerophosphoethanolamine, E'=glycerophosphocholine, F= phosphocreatine (PCr), G, H and K= γ, α , and β phosphorus atoms of nucleoside triphosphate(NTP), I = NAD(H) and J=diphosphodiester. Note that the spectra are stable over the duration of the study.

In contrast, figure 2 shows MDA-MB-435 cells treated with 6AN (200uM). The initial (pretreatment) spectra are similar to data shown in figure 1. Peak A = phosphogluconate which is produced from glucose-6-phosphate. The addition of 6AN leads to buildup of this peak which is known to be a glycolytic inhibitor. Treatment with 6AN also leads to a loss of PCr and NTP, an increase in Pi and splitting of the Pi resonance into two resolvable components, indicating a region of pH heterogeneity which likely represents the intra vs. extracellular (perfusion media) volume. The latter indicates that the cells became more acidotic (upfield shift) after treatment with 6AN. These findings are similar to our data with the MCF-7. Surviving fraction experiments to measure the effect of 6AN on cellular radiosensitivity and enhancement of the effect of adriamycin will begin shortly.

Preliminary experiments with the three drug regimen of PALA, MMPR and 6AN (referred to as MAP) administered prior to radiation were also performed. PALA was given at t=-17 hours, MMPR and 6AN at t=0 and XRT at t=10 hours. Mice treated with this regimen had 7/9 partial responses after 5 Gy and 7/7 responders after 10 Gy. After treatment with MAP→5Gy, the mice had not regrown to their initial pretreatment volume at the time of analysis (day 70) (Fig. 3A). After treatment with MAP→10 Gy, three of the 7 responders were complete responders, all of which remain in complete response after >60 days. In this group, the average pretreatment tumor volume was 148 +/- 8.2. On day 70, the average tumor was 18.6 +/- 7.5 with three of the mice having no visible tumor, and the tumor on the other 4 mice decreasing in size (Fig. 3B).

The data obtained in the MCF-7 tumor model thus indicate that 6AN is very effective in enhancing tumor response to radiation and also enhancing response to chemotherapy. This was the original

primary hypothesis to be tested. While the initial results are gratifying, particularly for radiation enhancement, the study on a hormone resistant model (which we have just begun), are particularly important, since enhanced systemic treatments for metastatic breast cancer are very important. The metabolic abnormalities detected in cell culture in the MDA-MB-435 cells indicate that there are 2 pH regions after treatment with 6AN. These two volumes are expected to be the perfusion media (pH should be invariant during the experiment) and the cells (more acid). The second Pi peak is indicative of acidosis and this suggests that tumor cells become both acidotic in addition to deprived of energy. The former may be useful since some the entry of some drugs into cells are pH dependent. This may be pursued separately if it can be reproduced in vivo.

It is also noted, that the enhanced response is associated with the use of tumor growth delay measurements, and also the more stringent requirement of tumor shrinkage. A major question about drug development and the relevance of animal studies is that many drugs are active in mice- far fewer are clinically active. The criterion for clinical activity (tumor shrinkage) is much more rigorous than for mice studies (tumor growth delay). Thus the fact that partial responses (tumor shrinkage) were noted in these studies, suggests that this drug has significant potential. Also since the question of timing is difficult to translate from mice to patients, the fact that drug metabolism can be monitored non-invasively using nuclear magnetic resonance spectroscopy, also will facilitate further clinical drug development.

Table 1 – Effect of 6AN on radiation response – Expt. 1

Treatment	Response (Time to regrowth (XRT) or Time to increase by 50% (6AN and controls)(days)
Control	20.6 +/- 4.7 days (Time to increase by 50%)
6AN	18.3 +/- 6.0 days (Time to increase by 50%)
5 Gy	32.0 +/- 4.3 (Time to regrow to initial volume)
6AN→5Gy	68.7 +/- 2.2 (Time to regrow to initial volume)

Table 2 - Effect of 6AN on radiation response – Expt. 2

Treatment Response

<u>Treatment</u>	<u>(Time to regrowth (XRT) or Time to increase by 50% (6AN and controls)(days)</u>
Control	13.3 +/- 3.4 days (Time to increase by 50%)
6AN	27.4 +/- 3.1 (Time to increase by 50%)
5 Gy	23.7 +/- 5.6 (Time to regrow to initial volume)
6AN→5Gy	55.0 +/- 6.4 (Time to regrow to initial volume)

Table 3 - Effect of 6AN on radiation response – Expt. 3

Treatment (Time to regrowth (XRT) or Time to increase by 50% (6AN and controls))(days)

Control	12.6 +/- 0.9 (Time to increase by 50%)
6AN	Not Done
5 Gy	18.7 +/-6.4 (Time to regrow to initial volume)
6AN→5Gy	39.7 +/- 7.1 (Time to regrow to initial volume)

Table 4 Combined Data from Tables 1-3

Treatment (Time to regrowth (XRT) or Time to increase by 50% (6AN and controls))(days)

Control	15.1 +/- 1.8 (n=24) (Time to increase by 50%)
6AN	24.0 +/- 3.0 (n=17) (Time to increase by 50%) *
5 Gy	23.7 +/- 5.6 (n=26) (Time to regrow to initial volume)
6AN→5Gy	53.7 +/- 4.0 (n=26) (Time to regrow to initial volume)**

* p=0.03 compared to control

** p<<0.001

Table 5 Effect of 6AN on Adriamycin Efficacy

Treatment	Days for tumor to increase by 50%
Control	9.4 +/-2.9 (n=19)
6AN	19.5 +/- 5.2 (n=15) (p<0.01 compared to control)
Adriamycin (10 mg/kg)	33.2 +/- 2.9 (n=12)
6AN→ Adriamycin (6mg/kg)	47.2 +/- 5.2** (n=14) (p<0.025 compared to Adriamycin)

MDA-MB-435 Cells – Control

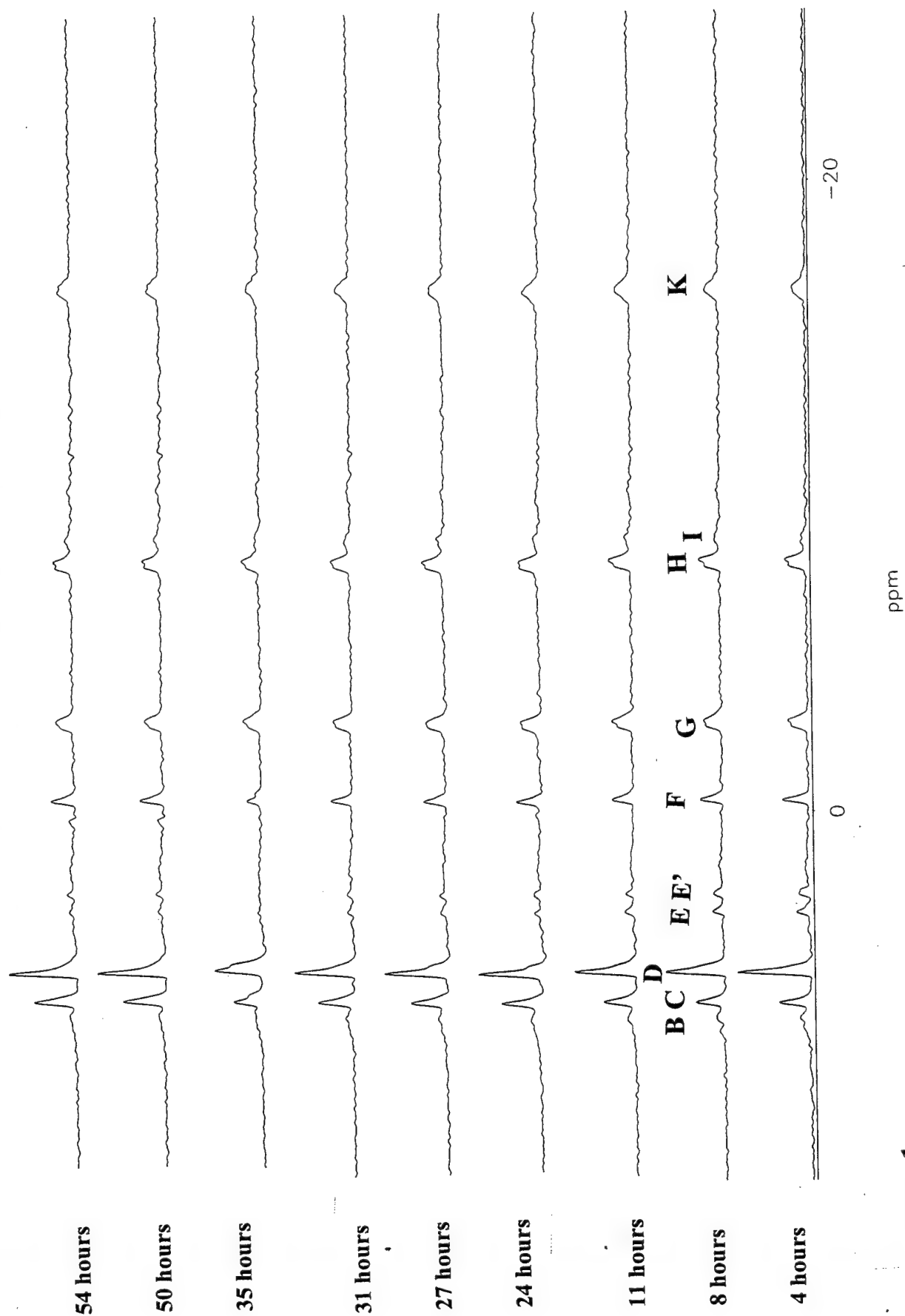


Figure 1

MDA-MB-435 Cells – 6AN (200uM, 4 hours)

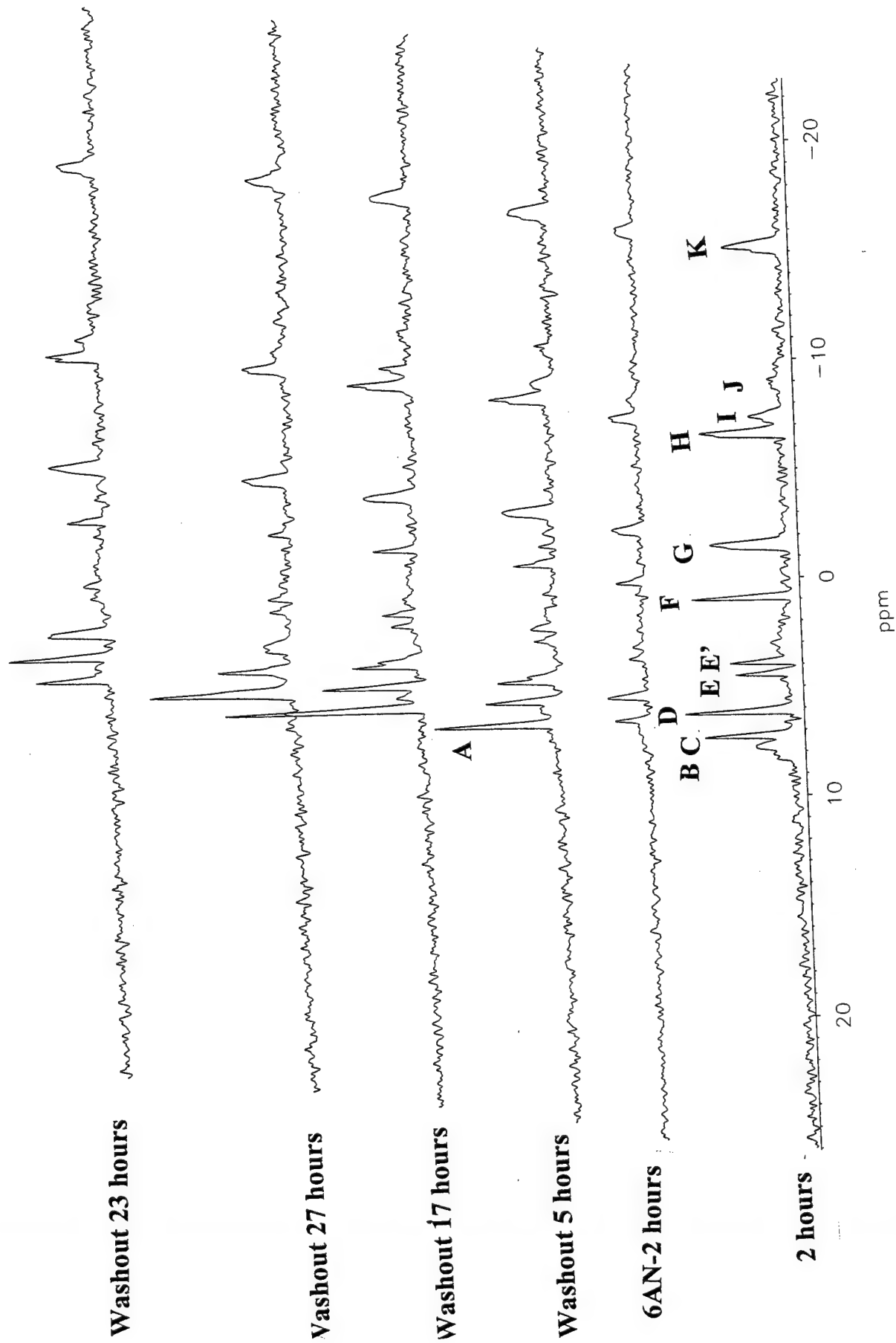


Figure 2

5 Gy Data

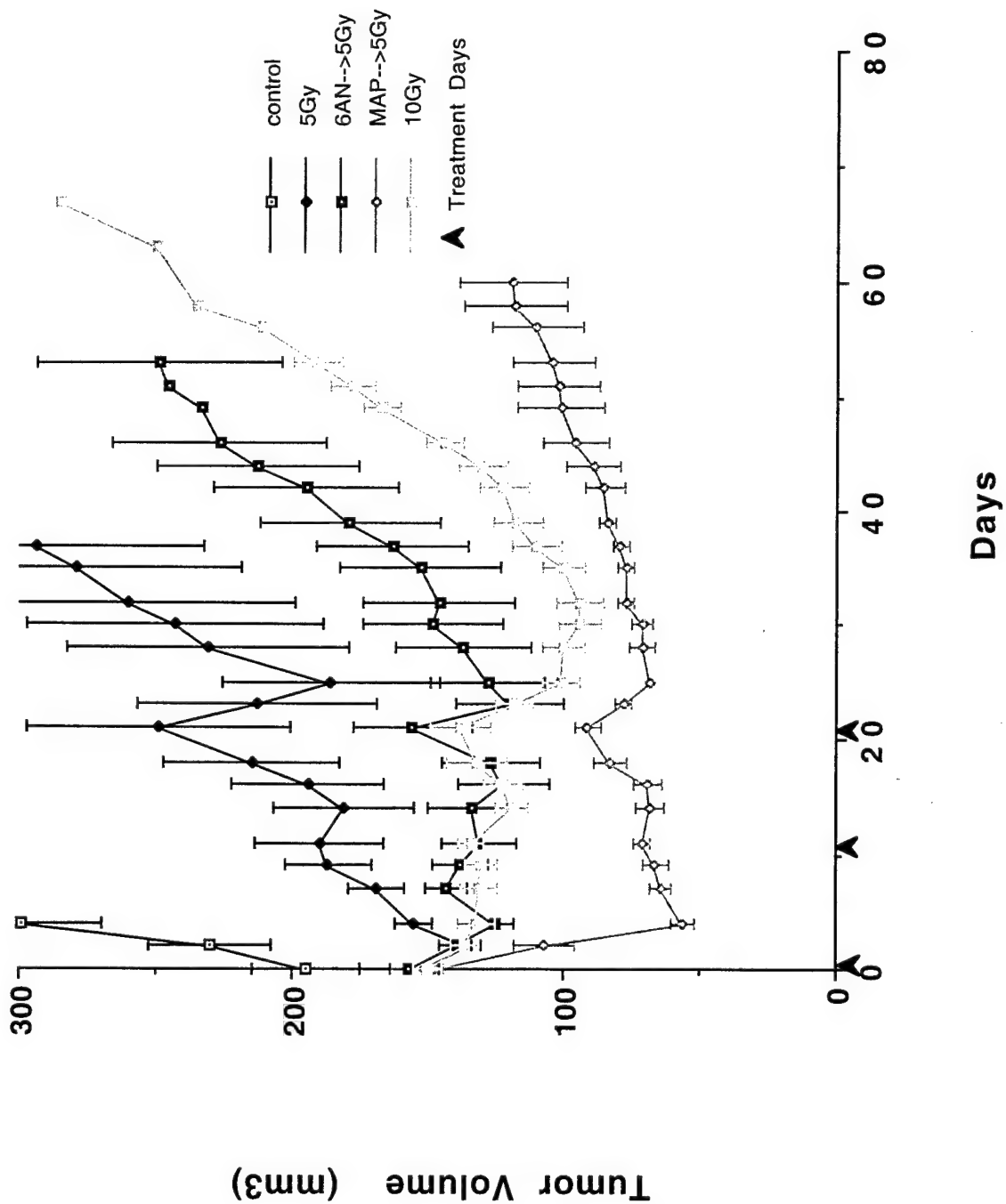


Figure 3A

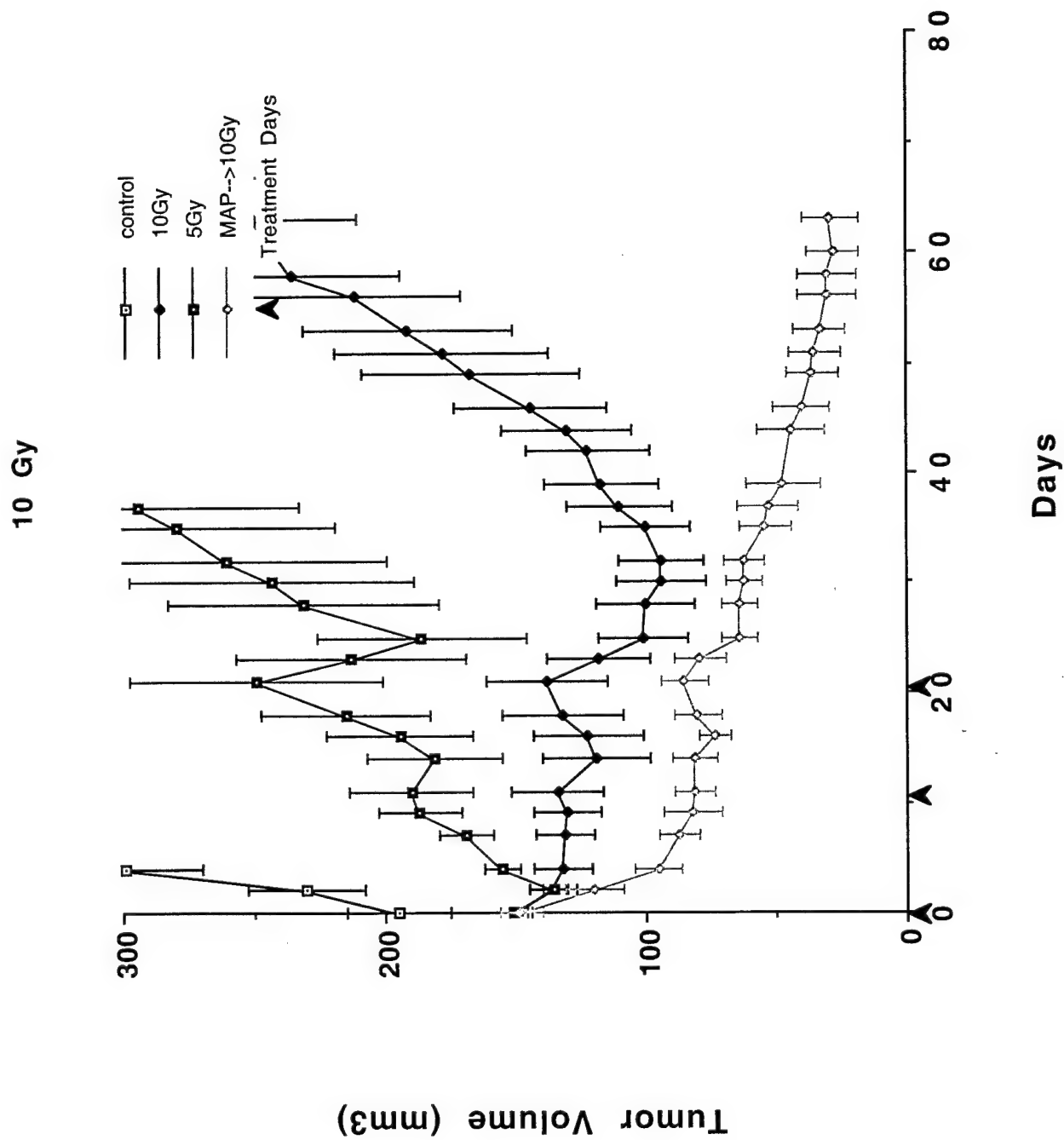


Figure 3B

Figure Legends

Figure 1 ^{31}P NMR spectra taken from perfused MDA-MB-435 cells. These are control spectra that demonstrate the stability of the system. Spectral peaks are B= phosphoethanolamine, C=phosphocholine, D=inorganic phosphate (Pi), E=glycerophosphoethanolamine, E'=glycerophosphocholine, F= phosphocreatine, G, H and K= γ, α , and β phosphorus atoms of nucleoside triphosphate, I = NAD(H) and J=diphosphodiester.

Figure 2. ^{31}P NMR spectra taken from MDA-MB-435 cells prior, during and after 4 hour exposure to 200 μM 6AN. The appearance of a new peak (A=phosphogluconate) is caused by treatment with 6AN. Also note the decrease in PCr and NTP and the splitting of the Pi peak into 2 components.

Figure 3 A. Effect of PALA, MMPR and 6AN on efficacy of 5 Gy. Note that by day 60, the tumor has not yet regrown to its pretreatment volume. Pretreatment with PALA, MMPR, 6AN (MAP) increases the efficacy of 5 Gy and the tumor regrowth is slower than after 10 Gy alone, indicating a dose modification factor of greater than 2 for MAP. B. Effect of MAP on tumor growth. Note that pretreatment with MAP has induced 3/7 complete responses (not shown) and the average tumor volume continues to decrease at >60 days.

Key Research Accomplishments

1. In a hormone sensitive human tumor (MCF-7), 6AN enhances the effect of radiation. This corroborates our previous results in a murine breast tumor. It is particularly noted, that this enhanced response is noted with the use of tumor growth delay measurements, and also the more stringent requirement of noting tumor shrinkage (as evidenced by partial responses).
2. In a hormone sensitive human tumor (MCF-7), 6AN enhances the effect of adriamycin. This enhancement is obtained without additional animal mortality and with a reduced dose of adriamycin.
3. In hormone resistant MDA-MB-435, we have been able to monitor the effect of 6AN on tumor metabolism
4. Early studies indicate that MAP is very effective in enhancing tumor response and can convert palliative response to radiation to complete responses. This suggests that previous studies in murine tumors (5) indicating that MAP is effective in inducing long term (>1 year) complete responses when combined with radiation may also be valid in human tumors.

Reportable Outcomes

Funding has been applied for based on this work:

1. Army Breast Cancer Grant – 2000
2. RAID grant (NIH) to proceed with obtaining clinical supplies of 6AN

Conclusions

In the MCF-7 tumor, tumor growth delay measurements and evaluation of tumor response indicate that 6AN is effective at enhancing response to radiation (5 Gy). There is also an enhanced response as measured by tumor growth delay for mice receiving 6AN→adriamycin compared to mice treated with Adriamycin alone. Preliminary studies with MAP and radiation indicate that MAP is very effective in enhancing tumor response and may convert palliative response to radiation to complete responses.

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